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Distinct, genome-wide, gene-specific selectivity patterns of four glucocorticoid receptor coregulators

Dai-Ying Wu, Chen-Yin Ou, Rajas Chodankar, Kimberly D. Siegmund and Michael R. Stallcup

Department of Biochemistry and Molecular Biology (D-Y W, C-Y O, RC, MRS), Department of Preventive Medicine (KDS), USC Norris Comprehensive Cancer Center, University of Southern California, Los Angeles, CA 90089

Footnotes: D-YW and C-YO are equal first authors. **Corresponding author:** MRS, stallcup@usc.edu

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Abbreviations: CCAR, cell cycle and apoptosis regulator; CoCoA, coiled-coil coactivator; CoR, coregulator; Dex, dexamethasone; DMEM, Dulbecco's Modified Eagle Medium; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GR, glucocorticoid receptor; H3K4me1, histone H3 monomethylated at Lys-4; H3K4me3, histone H3 trimethylated at Lys-4; H3K9ac, histone H3 acetylated at Lys-9; H3K9me3, histone H3 trimethylated at Lys-9; H3K27ac, histone H3 acetylated at Lys-27; H3K27me3, histone H3 trimethylated at Lys-27; IPA, Ingenuity Pathway Analysis; JAK, Janus kinase; JNK, Jun kinase; MAP kinase, mitogen activated protein kinase; NFκB, nuclear factor KB; NS, non-specific; STAT, signal transducer and activator of transcription; TNF, tumor necrosis factor; TNFR2, TNF receptor 2; TSS, transcription start site; *ZNF282*, zinc finger protein 282.

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Glucocorticoids are a class of steroid hormones that bind to and activate the glucocorticoid receptor (GR), which then positively or negatively regulates transcription of many genes that govern multiple important physiological pathways such as inflammation and metabolism of glucose, fat and bone. The remodeling of chromatin and regulated assembly or disassembly of active transcription complexes by GR and other DNA-binding transcription factors is mediated and modulated by several hundred transcriptional coregulator proteins. Previous studies focusing on single coregulators demonstrated that each coregulator is required for regulation of only a subset of all the genes regulated by a steroid hormone. We hypothesized that the gene-specific patterns of coregulators may correspond to specific physiological pathways such that different coregulators modulate the pathway-specificity of hormone action, thereby providing a mechanism for fine tuning of the hormone response. We tested this by direct comparison of multiple coregulators, using siRNA to deplete the products of four steroid hormone receptor coregulator genes (*CCAR1*, *CCAR2*, *CALCOCO1* and *ZNF282*). Global analysis of glucocorticoid-regulated gene expression after siRNA mediated depletion of coregulators confirmed that each coregulator acted in a selective and gene-specific manner and demonstrated both positive and negative effects on glucocorticoid-regulated expression of different genes. We identified several classes of hormone-regulated genes based on the effects of coregulator depletion. Each coregulator supported hormonal regulation of some genes and opposed hormonal regulation of other genes (coregulator-modulated genes), blocked hormonal regulation of a second class of genes (coregulator-blocked genes), and had no effect on hormonal regulation of a third gene class (coregulator-independent genes). In spite of previously demonstrated physical and functional interactions among these four coregulators, the majority of the several hundred modulated and blocked genes for each of the four coregulators tested were unique to that coregulator. Finally, pathway analysis on coregulator-modulated genes supported the hypothesis that individual coregulators may regulate only a subset of the many physiological pathways controlled by glucocorticoids. We conclude that gene-specific actions of coregulators correspond to specific physiological pathways, suggesting that coregulators provide a potential mechanism for physiological fine tuning *in vivo* and may thus represent attractive targets for therapeutic intervention.

Introduction

Nuclear receptors are ligand-regulated transcription factors through which the cell responds to external stimuli. They can detect the presence of a small molecule ligand (e.g. a hormone, vitamin or metabolite) and modify cellular gene expression to respond accordingly. The steroid hormone

receptors,including the receptors for estrogens, progestins, androgens, glucocorticoids, and mineralocorticoids, form one class of nuclear receptors. Canonical steroid receptor function involves the receptor binding to its ligand, which alters receptor conformation and potentiates binding to a specific related set of DNA motifs that serve as regulatory elements for specific genes. The DNA-bound receptors recruit a large number of transcriptional coregulator proteins, which remodel chromatin and regulate the assembly or disassembly of active transcription complexes on the transcription start sites of the genes associated with the enhancer and silencer elements. Coregulators are essential for proper gene regulation, and coregulator mutants are involved in several diseases [1].

Glucocorticoid receptor (GR, official symbol NR3C1) is activated in humans by the steroid hormone cortisol, which is produced in the adrenal cortex in response to many types of stress and serves a homeostatic function by regulating many different physiological pathways. Synthetic glucocorticoids, such as dexamethasone (dex), are one of the most widely prescribed classes of drugs, used clinically for their anti-inflammatory and immune-suppressive effects and in some cancer chemotherapy regimens. They are highly effective but have a host of deleterious side effects such as weight gain, insulin resistance, hyperglycemia, hyperlipidemia, osteoporosis, and muscle wasting [2–4]. This reflects the role of glucocorticoids in regulating inflammation and immune response, as well as metabolism of glucose, lipids, and bone, among other physiological pathways.

A number of recent studies, each focusing on a single coregulator, indicated that steroid receptor coregulators function in a gene-specific manner and are required for regulation of only a subset of the genes activated or repressed by a steroid hormone and its receptor [1, 5–10]. This invites the hypothesis that different coregulators could regulate different physiological pathways controlled by glucocorticoids [11, 12]. Such a hypothesis necessitates that different coregulators are required for hormonal regulation of different sets of genes. However, direct comparisons of the gene-specific actions of multiple coregulators for a specific steroid receptor in a single cell line have yet to be reported. To test this hypothesis, we conducted an unbiased, genome-wide analysis of the effects of depleting four different coregulators on glucocorticoid-regulated gene expression in the A549 lung adenocarcinoma cell line. We expected to find different but overlapping subsets of genes that are controlled by each coregulator, and we used pathway analysis to test whether these gene subsets represent different known physiological pathways that are regulated by glucocorticoid hormone.

The four nuclear receptor coregulators used in this study were chosen based on known physical and functional interactions and some structural homology. *CCAR1* (cell cycle and apoptosis regulator 1, also known as CARP1), is important for cell cycle regulation and binds to nuclear receptors in a hormone dependent manner [5]. *CCAR2* (also known as deleted in breast cancer 1, DBC1, or KIAA1967) is a paralog of *CCAR1* and has been shown to work synergistically with *CCAR1* in a hormone dependent manner to coactivate target gene expression [6]. CoCoA (coiled-coil coactivator, gene name *CALCOCO1*) has a coiled-coil domain, binds the p160 coactivator complex and enhances transcriptional activation of nuclear receptors [7]. Lastly *ZNF282* (homolog of Zfp282 in mice and rats, also known as HUB1), has been shown to bind and coactivate estrogen receptor and function synergistically with CoCoA [8]. Structurally, *ZNF282* has five C2H2 zinc fingers that can bind DNA along with a repressive KRAB domain [8]. *CCAR1*, *CCAR2*, and *ZNF282* can bind to the C-terminal activation domain of CoCoA. Several combinations of these coregulators act cooperatively to enhance steroid receptor activity in transient reporter gene assays [1, 5–10]. We thus proposed to test whether the physical and functional relationships among these four coregulators might result in substantial overlap in the subsets of glucocorticoid-regulated genes and the corresponding physiological pathways they control.

Materials and Methods

Cell culture and RNA interference

A549 human lung carcinoma cells were purchased from the American Type Culture Collection and grown in DMEM with 10% fetal bovine serum (FBS) at 37℃ in a humidified incubator with 5% CO2. For coregulator depletion, siRNA was transfected into A549 cells using Oligofectamine according to the manufacturer's protocol. Two days after transfection, the media was changed to hormone-free medium (phenol-red free DMEM) supplemented with 5% charcoal-stripped FBS. The next day, cells were subjected to hormone treatment with 100 nM dex or ethanol as control for 6 hrs. For immunoblot assay, cells were harvested right before hormone treatment in RIPA buffer supplemented with protease inhibitor cocktail tablets (Roche). For RT-PCR assay, cells were collected 6 hours after hormone treatment. The sequences of the sense siRNAs used are as follows: nonspecific (NS) control siRNA [10]; si-*CCAR1*, 5'-GCCCTAGTATGGAAGATTT-3'; si-CoCoA [7]; si-*CCAR2* [13]; si-*ZNF282* [8]. The siRNAs for *CCAR1* [14], *CCAR2* [6],CoCoA [15], and *ZNF282* [8] were each previously validated with a second siRNA designed against a different part of the same mRNAs to demonstrate that their effects were specific and not off-target effects.

Immunoblot analysis

Collected cell lysates were subjected to centrifugation for 15 min at the maximum speed of a microcentrifuge at 4℃. The supernatant was resolved by SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed with primary antibodies against the following proteins: β-actin (Sigma); *ZNF282* (Sigma); *CCAR1* (Bethyl Laboratory); *CCAR2* (Bethyl Laboratory); CoCoA (Bethyl Laboratory).

Quantitative reverse transcriptase-PCR

For RT-qPCR assay, RNA was extracted from cells after 6 hr of hormone treatment using the RNeasy kit (Qiagen). cDNA was synthesized by reverse transcribing 0.9 µg of total RNA using iScript cDNA synthesis kit (Bio-Rad). The cDNA was mixed with appropriate primers and LightCycler 480 SYBR Green I Master (Roche), and the mixture was then analyzed with the LightCycler 480 System (Roche). The primers used are as follows: β-actin [16]; PPM1E,5'-AGAGCCACATCAGATGAAGTCC-3' (forward) and 5'-ACGGGCCAATTTCACTGTCTC-3' (reverse); and AP5P1, 5'-GGGAGCGTAGCCTTACAGC-3' (forward) and 5'-AGTGAGCAGATAGGAGGTGTC-3' (reverse).Results shown are mean and range of variation for duplicate PCR reactions from a single cDNA preparation, and are representative of a minimum of four independent experiments. Relative mRNA expression levels were determined by normalizing against β-actin mRNA.

Microarray analysis

We used an Illumina HT12v4 microarray to interrogate the genome-wide mRNA levels 6 hours after treatment of A549 cells with ethanol control or 100 nM dex, using cells that were previously transfected with a control siNS (non-specific sequence) or siRNA directed against CCAR1, CCAR2, CoCoA, or ZNF282. All experiments had 4 biological replicates performed on different days except our siNS control (6 replicates) and siZNF282 (2 replicates). Analysis of the microarray data was performed using bioconductor package beadarray (v2.8.1) [17,18] to process the raw data and filter non-specific probes followed by limma (v3.14.4) [24] to identify differentially expressed genes.

The microarray has over 45k probes with most genes represented by a single probe, thus the term gene was used when describing numbers of significant probes found from the microarray. We performed and overlapped three two-way comparisons to identify hormone-regulated genes in cells transfected with siNS (Figure 1a, comparison 1), hormone-regulated genes in coregulator depleted cells (Figure 1a, comparison 2), and genes affected by coregulator depletion in hormone-treated cells (Figure 1a, comparison 3). We used q-value to estimate the false discovery rate and account for multiple hypothesis testing; calculations were done using [25], and we called a difference in gene expression significant if there was greater than 1.5-fold change and q-value of < 0.05. The processed data can be found in the spreadsheet in Supplementary Material and the raw data can be found under GEO accession number GSE58715. Weighted venn diagrams were based on weights calculated from eulerAPE [21]. We used IPA (Ingenuity Systems, www.ingenuity.com) to identify pathways affected by each of the gene sets that we identified. Modulated genes were identified using a significant cutoff of q-value < 0.05 without fold-change cutoff.

Results

Coregulator depletion alters hormonal regulation of subsets of genes

To evaluate the relationship between coregulator and hormone, we identified and overlapped a set of hormone-regulated genes with no coregulator depletion (Figure 1a, comparison 1), hormone-regulated genes upon coregulator depletion (Figure 1a, comparison 2), and coregulator-regulated genes, i.e. genes from dex-treated cells with different mRNA levels when comparing cells containing and lacking a coregulator (Figure 1a, comparisons 3). From these comparisons, we identified dex-regulated genes that are not significantly affected by coregulator depletion (Figure 1b compartment i), genes affected by coregulator depletion but not significantly hormone-regulated (Figure 1b ii), genes that remained hormone-regulated after coregulator depletion (Figure 1b v, vii), hormone-regulated genes that were affected by coregulator depletion (Figure 1b iii, vii) and genes that gained hormone regulation upon coregulator depletion (Figure 1b iv, vi). In our detailed analysis below, we do not focus on genes that have similar gene expression levels after hormone treatment (Figure 1b iv) and that gain hormone regulation upon coregulator depletion because this situation occurs with changes of gene expression in the absence of hormone caused by coregulator depletion (baseline differences). In general, we found fewer hormone-regulated genes after coregulator depletion (comparison 2 smaller than comparison 1), and thousands of genes were affected by the depletion (comparison 3), with *ZNF282* depletion having the largest effect with almost 3000 genes affected while the other coregulator depletions have around 1100 genes affected (Figure 1c-f).

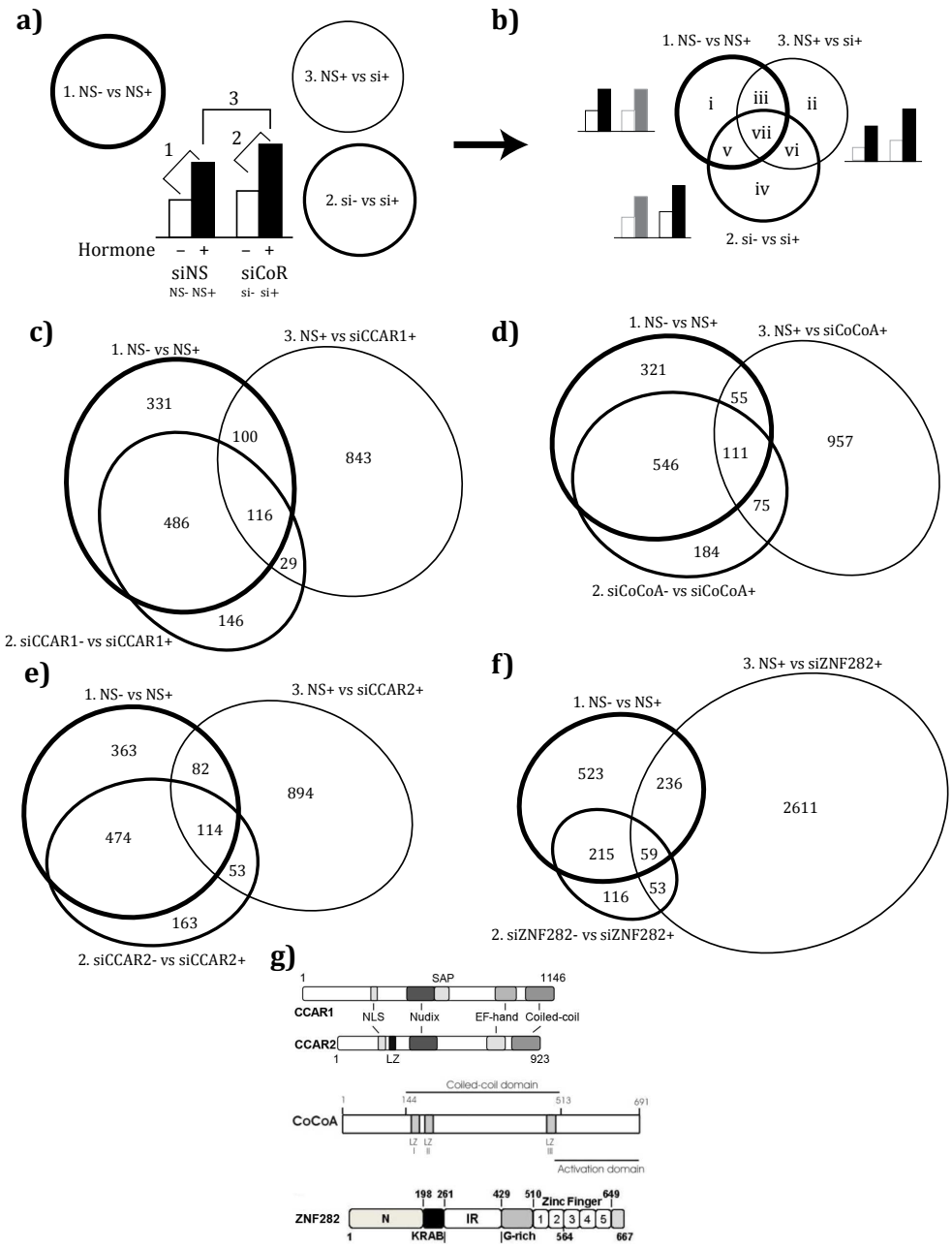

Figure 1. Effect of hormone and coregulator depletion on gene expression. (a) For each coregulator, A549 cells were transfected with coregulator-specific (siCoR) or control non-specific (siNS) siRNA, and cells were subsequently treated with dex or ethanol for 6 hours. Genome-wide microarray analyses of RNA with multiple independent biological replicates of each condition were conducted. The 3 comparisons of interest are: comparison 1, cells transfected with siNS and then treated with dex or ethanol (NS- vs NS+); comparison 2, cells transfected with siCoR and then treated with dex or ethanol (si- vs si+); comparison 3, cells transfected with siNS or siCoR and then treated with dex (NS+ vs si+). (b) Venn diagrams are used to show the overlap between significant genes from the three comparisons. Overlapping compartments are labeled for convenient reference. For each comparison, the theoretical data bars representing relevant expression values being compared are shown in black beside the Venn diagram, while the bars representing expression values that are not part of the comparison are shown in gray. (c-f) As explained in (a) and (b), venn diagrams were created from the overlapping statistically significant effects of hormone and coregulator depletion to visualize the number of genes affected by depletion of CCAR1 (c), CoCoA (d), CCAR2 (e), and ZNF282 (f). The size of each ellipse and overlap compartment in the Venn diagram is proportional to the number of genes affected. We called a difference in gene expression significant if there was greater than 1.5-fold change and a false discovery q-value < 0.05. (g). Domains of the four coregulators.

Except for *ZNF282*, the majority of hormone-regulated genes remained hormone-regulated after coregulator depletion (Figure 1b compartments v + vii are large compared to iv + vi or i + iii). Of the genes regulated by dex either in the presence (comparison 1) or absence (comparison 2) of coregulator, we found around 250 genes with their dex-regulated level of expression altered by depletion of *CCAR1* (245), CoCoA (241), or *CCAR2* (249), while 348 genes were affected by depletion of *ZNF282* (Figure 1b iii, vi, vii). These genes correspond to about one fifth of all hormone-regulated genes (*CCAR1*: 254 / 1208 = 0.20, CoCoA : 241 / 1292 = 0.19, *CCAR2* : 249 / 1249 = 0.20, *ZNF282*: 348 / 1202 = 0.29, Figure 1b iii + vii + vi / all but ii). Additionally, with the exception of CoCoA, there were more coregulator-regulated genes that lost hormonal regulation upon coregulator depletion (Figure 1b iii) than genes that newly acquired hormonal regulation after depletion of coregulator (Figure 1b vi) (iii vs. vi: 100 vs. 29 for *CCAR1*, 55 vs. 75 for CoCoA, 82 vs. 53 for *CCAR2*, 236 vs. 53 for *ZNF282*). Lastly, of the 1033 hormone-regulated genes (comparison 1), 53% were affected by depletion of one or more of the four coregulators studied (unique genes in the sum of compartments iii + vii for all four coregulators).

The fact that *ZNF282* depletion altered expression of a much larger number of genes than depletion of the other three coregulators (Figure 1f comparison 3) and reduced the number of hormone-regulated genes more than did depletion of the other three coregulators (Figure 1f comparison 2 vs. comparison 1) suggests that *ZNF282* may have a greater impact on gene regulation than the other three coregulators. Using no fold change cutoff (instead of the 1.5-fold cutoff used in Figure 1), we found similar patterns of overlaps (Supplementary File 1). When a more stringent 2-fold change cutoff was applied, most hormone-regulated genes were still shared in comparisons 1 and 2 (compartments v + vii), but a lower percentage of coregulator-regulated genes were hormone-regulated (Supplementary File 2, compartments iii + vii). We concluded from this comparison that the pattern of overlaps between the different coregulators remains consistent with different fold change cutoffs, with higher fold-change cutoffs detecting fewer coregulator effects.

Since this study analyzed the roles of coregulators in dex-regulated gene expression, the subsequent sections focus on a subset of genes located in the compartments where effects of coregulator depletion in comparison 3 overlap with dex-regulated comparison 1 or 2 (compartments iii, vi, and vii). We defined as "coregulator-modulated genes" those in compartments iii and vii, i.e. genes that were regulated by hormone in the presence of coregulator (comparison 1) and regulated by coregulator in the presence of dex (comparison 3). Among modulated genes, compartment iii contains genes that lose significant regulation by dex after coregulator depletion, while compartment vii represents genes that are significantly dex-regulated in the presence or absence of coregulator, but whose mRNA level in the presence of dex was significantly altered by coregulator depletion. We defined "blocked genes" as those in compartment vi, i.e. genes that only become dex-regulated after coregulator depletion and whose mRNA level in the presence of dex was significantly altered by coregulator depletion. Blocked genes indicate a novel and perhaps unexpected type of coregulator function where the coregulator was blocking the hormone response for genes in this class. We found several hundred modulated genes for each coregulator (*CCAR1*: 216, CoCoA: 166, *CCAR2*: 196, *ZNF282*: 295) and a smaller number of blocked genes (*CCAR1*: 29, CoCoA: 75, *CCAR2*: 53, *ZNF282*: 53). Gene expression changes and genes belonging to these two classes can be found in the spreadsheet in Supplementary Material.

Modulated and blocked regulatory genes are often unique to each coregulator

To evaluate coregulator specificity, we overlapped the genes found for each coregulator in each of the two gene classes of interest to look for shared regulation between different coregulators. Modulated genes (Figure 2a) have some overlap between the different coregulators; however, about one third of the genes were unique to each depleted coregulator (*CCAR1*: 38%, CoCoA: 31%, *CCAR2*: 32%, *ZNF282*: 39%) with about another third shared with one other coregulator (*CCAR1*: 38%, CoCoA: 36%, *CCAR2*: 39%, *ZNF282*: 38%) and the remaining genes shared by two or three coregulators. Blocked genes (Figure 2b) were nearly all unique to each coregulator, indicating that this type of function was highly specific for each coregulator. The largest overlap in blocked genes occurred between CoCoA blocked genes and *CCAR2* blocked genes with 8 genes shared (out of 53 genes for *CCAR2* and 75 genes for CoCoA). Surprisingly, the high structural homology between *CCAR1* and *CCAR2* (Figure 1g) and the previously reported physical and functional interactions among these four coregulators do not lead to substantial proportions of modulated and blocked genes overlapping between the various pairs of proteins.

Blocked genes have similar chromatin profiles to hormone-regulated genes

To investigate blocked genes, we scanned the transcription start site (TSS) for various activating and repressive histone marks. We wished to look for patterns in the chromatin structure that might give insight into mechanism of regulation for blocked genes. We used publically available ENCODE histone ChIP-seq data for the A549 cell line and scanned a window of -100 bp to +10 bp around the TSS to identify the significant histone marks. We evaluated

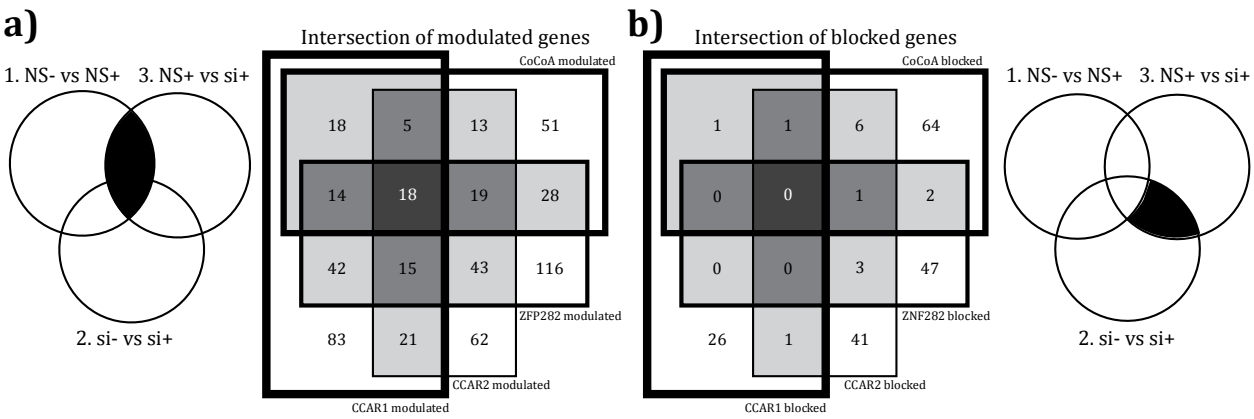

Figure 2. Four-way Venn diagrams are used to indicate the overlap of the dex-regulated genes that belong to the modulated (a) or blocked (b) gene sets for each of the four coregulators. Numbers indicate number of genes. The darkest shading indicates genes that are common to all four coregulators, and the unshaded regions indicate genes that are uniquely regulated by a single coregulator. The dark areas highlighted in the accompanying three-way venn diagrams indicate how the modulated and blocked gene sets were derived from Figure 1.

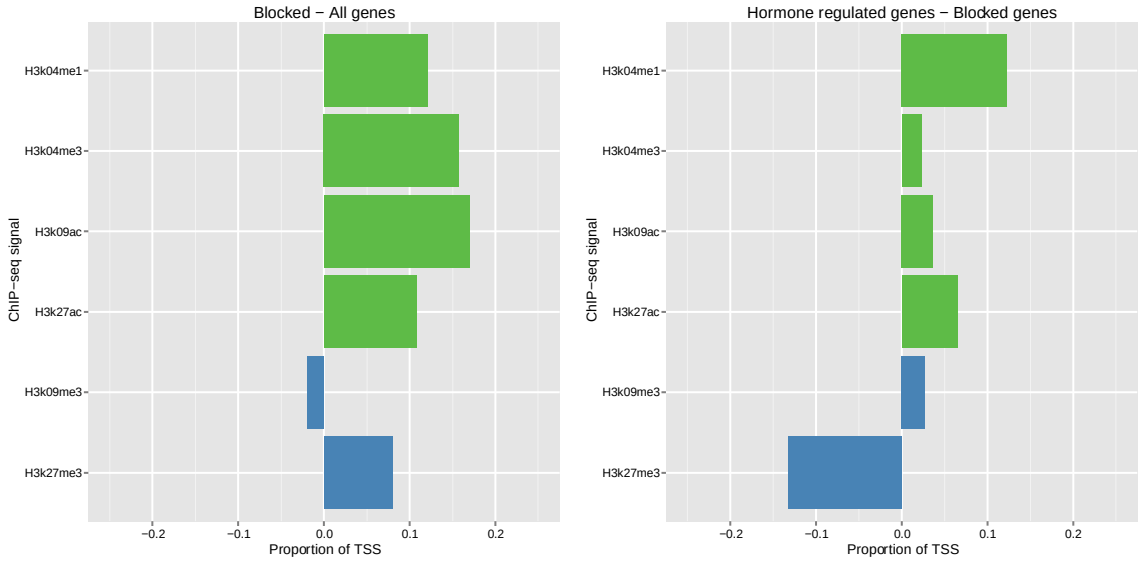

Figure 3. Comparison of the fraction of histone marks that cover the transcription start sites (TSS) of blocked genes, hormone-regulated genes and all genes. a) Differences in proportion of each histone mark at TSS of blocked genes versus TSS of all genes on microarray. b) Differences in proportion of each histone mark at TSS of hormone-regulated genes versus blocked genes.

the presence of 4 activating histone marks (H3K4me1, H3K4me3, H3K9ac, H3K27ac) and 2 repressive marks (H3K9me3, H3K27me3) around the TSS of 193 different blocked genes, 1033 hormone-regulated genes, and all genes (Supplementary Files S3-6). We found differences in the proportion of histone marks at TSS of blocked genes compared to all genes with about 15% more H3K4me3, and H3K9ac in blocked genes (Figure 3 left). Comparing the proportion of histone marks at dex-regulated genes to blocked genes (Figure 3 right), we found similar proportions of some histone marks (H3K4me3, H3K9ac, H3K9me3) while H3K4me1 was more prevalent in hormone-regulated genes and H3K27me3 was more prevalent in blocked genes. We speculate that although blocked genes share many chromatin features with hormone-regulated genes, their hormone regulation was blocked by repressive histone marks that were somehow facilitated by a coregulator.

Most blocked genes are up-regulated by hormone

To characterize the gene selective action of activation and repression for each coregulator, we analyzed the direction of regulation of blocked genes. Blocked

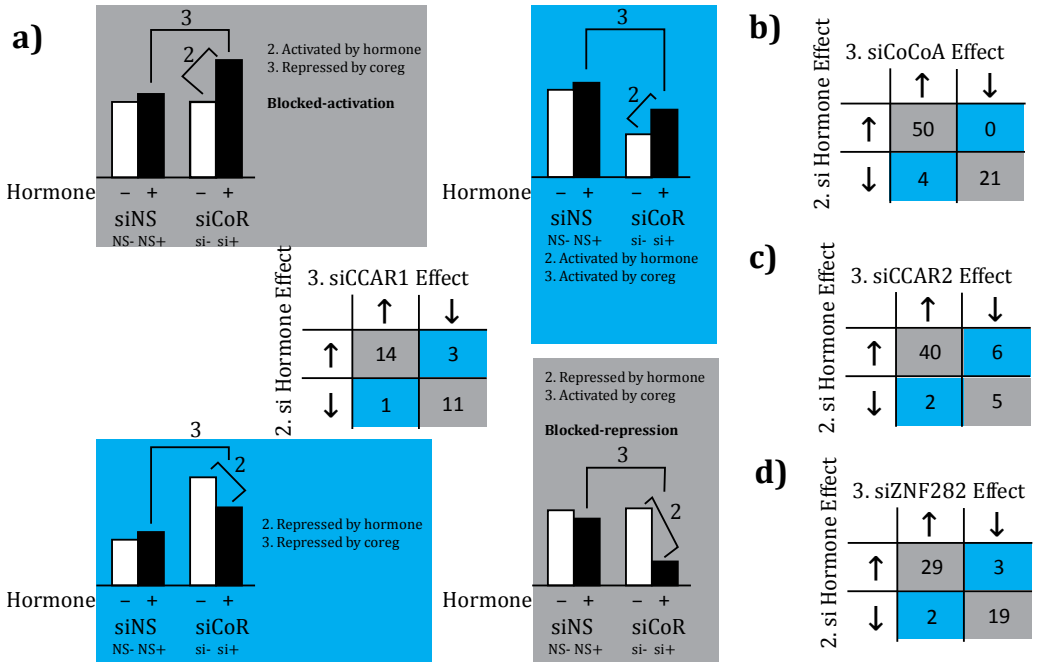

Figure 4. Tables showing the direction of regulation by hormone treatment and by coregulator depletion for coregulator-blocked genes. Four possibilities exist depending on direction of change from hormone treatment and coregulator depletion. Theoretical examples to illustrate data for each type of regulation are shown along with breakdown of the number of genes in each category for CCAR1 depletion (a), CoCoA depletion (b), CCAR2 depletion (c), and ZNF282 depletion (d).

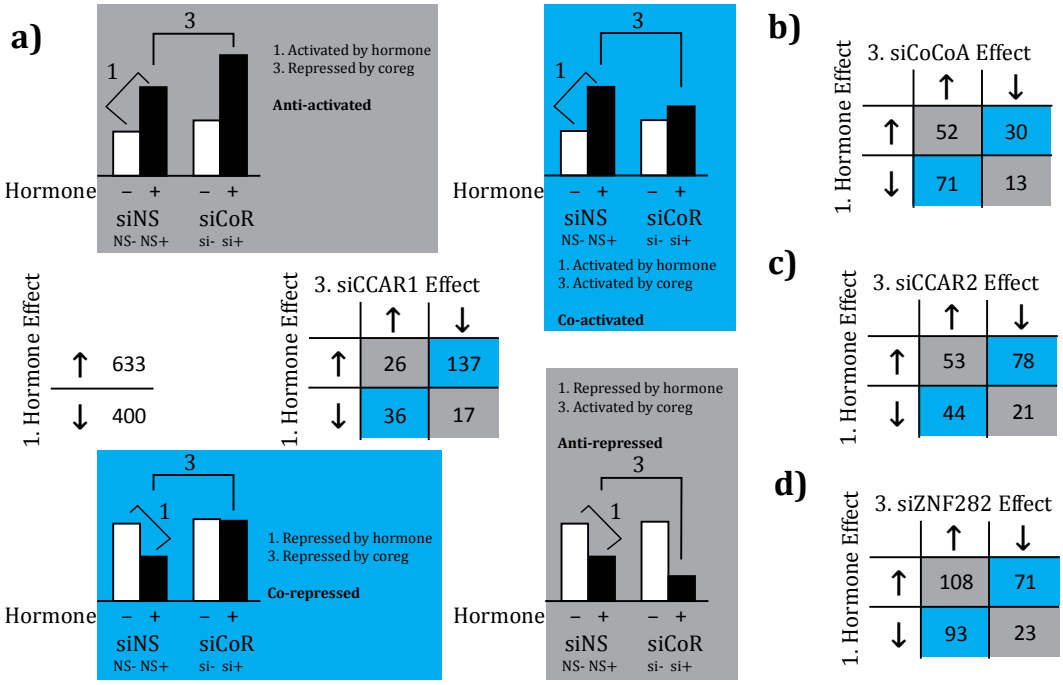

Figure 5. Tables showing the direction of regulation by hormone treatment and by coregulator depletion for coregulator-modulated genes. Four possibilities exist depending on fold change from hormone treatment and coregulator depletion. Theoretical examples to illustrate data for each type of regulation are shown along with breakdown of the number of genes in each category for CCAR1 depletion (a), CoCoA depletion (b), CCAR2 depletion (c), ZNF282 depletion (d).

genes were not significantly dex regulated in the presence of coregulator but become significantly dex-regulated after coregulator depletion, and their level of expression in the presence of dex was significantly altered by coregulator depletion (Figure 1b compartment vi). In Figure 4a, we show theoretical examples of gene expression changes indicative of coregulator-blocked activation (upper-left) and coregulator-blocked repression (lower-right) – these were the predominant categories of blocked genes, where the presence of the coregulator prevented hormone regulation. The remaining two comparisons (upper-right and lower-left) required changes to basal gene expression levels prior to hormone treatment due to coregulator depletion (baseline differences) and represent only a small percentage of the blocked genes (CCAR1: 4 genes (15%), CoCoA: 4 genes (5%), *CCAR2*: 8 genes (15%), and *ZNF282*: 5 genes (9%)).

Blocked activation (Figure 4a, upper left) was the most common type of regulation among the blocked genes for these four coregulators with 75% of *CCAR2*, 67% of CoCoA, 55% of *ZNF282*, and 48% of *CCAR1* blocked genes in this category. This category represents coregulators repressing hormone activation at these genes. After verifying that each coregulator was effectively depleted by its respective siRNA (Supplementary File 7), we validated selected blocked genes (*PPME1*, blocked by *CCAR1*, and AP5P1, blocked by *ZNF282*) by qPCR (Supplementary File 8). Among all dex-regulated genes, some were very highly regulated by dex; in contrast, changes for blocked genes were all less than four-fold (Supplementary File 9).

Coregulators both support and antagonize the hormone effect on modulated genes in a gene-specific manner

Next we investigated the direction of regulation of coregulator-modulated genes. Modulated genes were defined as dex-regulated in the presence of coregulator, and their level of expression in the presence of dex was altered by coregulator depletion (Figure 1b compartment iii and vii). Our work generalized the idea that each coregulator can function as either a coactivator or corepressor and can support or oppose the regulation by hormone. However, the ratio of genes that were positively or negatively regulated was unique to each coregulator (Figure 5, ratio between numbers in table for each coregulator). We define coactivated genes as being activated by hormone and requiring the presence of the coregulator for activation, corepressed genes as being repressed by hormone and requiring the presence of the coregulator for repression, anti-activated genes as being activated by hormone but repressed by coregulator, anti-repressed genes as being repressed by hormone but activated by coregulator. In Figure 5a, we show theoretical examples of gene expression patterns for coactivated (upper-right) and corepressed (lower-left) genes – coregulator activity in the same direction as dex hormone – while anti-activated (upper-left) and anti-repressed (lower-right) genes show coregulator activity that opposes the direction of dex regulation.

All four of the coregulators that we depleted have been shown previously to have coactivating activity for selected target genes in ligand-activated nuclear receptor systems [5–8]. We found that *CCAR1* modulated genes were mostly coactivated with *CCAR1* supporting the direction of dex regulation. In contrast, the predominant effects of CoCoA and *ZNF282* depletion on dex-regulated gene expression was negative, with *ZNF282* having an anti-activating effect on many genes. One reason *ZNF282* could be having a greater repressive effect compared to the other coregulators was because of its repressive KRAB domain [8]. The structurally-related proteins *CCAR1* and *CCAR2* share a similar direction of regulation for the modulated genes, with the majority (80% for *CCAR1*, 62% for *CCAR2*) of genes regulated by the coregulator in the same direction as dex. Over 60% of modulated genes were up-regulated by dex (except for CoCoA which has 49%), and the anti-repressed effect was the least common type of gene regulation by all coregulators (8% for *CCAR1*, 8% for CoCoA, 11% for *CCAR2*, 8% for *ZNF282*).

Coregulator specificity in differential regulation of physiological pathways

Glucocorticoids regulate many different developmental, metabolic, and inflammatory pathways and mediate responses to various types of stress, including hunger, cold, anxiety, and disease. The gene-specific actions of coregulators, as documented above, provide opportunities for the cell to modulate the specific genes that are regulated by glucocorticoids, through regulation of coregulator protein levels or regulation of coregulator activity via protein-protein interactions or post-translational modifications. To test whether coregulator gene-specificity is associated with specific glucocorticoid-regulated physiological pathways, we performed Ingenuity Pathway Analysis (IPA) on the coregulator-modulated gene set for each of the four coregulators examined above. We focused on the anti-inflammatory actions of glucocorticoids, since these complex pathways are key regulatory targets of GR. In addition, glucocorticoid regulation of many anti-inflammatory genes is common to a wide variety of cell types, including the A549 cell line used for this study, whereas other glucocorticoid-responsive metabolic pathways may be more tissue specific. IPA canonical pathway analysis found several inflammatory pathways that were enriched among dex-regulated genes and in one or more of the four coregulator-modulated gene sets under investigation in this study. Here we focus our discussion on the tumor necrosis factor receptor 2 (TNFR2), acute

phase, and interferon signaling pathways, which provide examples of both shared and coregulator-specific regulation of specific physiological pathways.

The TNFR2 pathway mediates signaling for TNFα/β and includes the well known NFκB inflammatory pathway as well as the JUN kinase (JNK) pathway, which is one of the three mitogen activated protein kinase (MAP kinase) pathways [22]. In A549 cells dex down-regulated several genes in both the NFκB and JNK pathways, as expected since glucocorticoids are anti-inflammatory (Supplementary File 10). Depletion of ZNF282, CoCoA and CCAR2 increased expression of several dex-regulated NFκB pathway genes, indicating that these three coregulators support the down-regulation of this inflammatory pathway (Figure 6). *ZNF282* was also required for the down-regulation by dex of a key transcription factor, c-Jun, at the distal end of the JNK pathway. In contrast, *CCAR1* depletion had no effect on genes in the NFκB pathway and had a mixed effect on the JNK pathway. A statistical analysis by IPA of the predicted effect of each coregulator on the TNFR2 pathway indicated highly significant effects by CoCoA and *ZNF282* but non-significant effects for *CCAR1* and *CCAR2* (Figure 6).

The acute phase response is a systemic defense system that responds to infection and stress and helps to prevent infection and initiate inflammatory processes [14]. This pathway contains both the NFκB and JNK pathways controlled by TNFR2 but also includes the ERK and p38 MAP kinase pathways and the STAT3 pathway. In addition to down-regulating components of the NFκB and JNK pathways, dex also up-regulates STAT3 in A549 cells (Supplementary File 11a). Depletion of *ZNF282* and *CCAR2* blocked dex inhibition of the NFκB pathway, and depletion of *ZNF282* and CoCoA enhanced dex stimulation of STAT3 expression, whereas neither *CCAR1* nor *CCAR2* affected STAT3 expression in dex-treated A549 cells (Supplementary File 11b-e). In contrast to the different pathway specificities of the four coregulators in the TNFR2 and acute phase response pathways, all four coregulators had similar effects on components of the interferon pathway in A549 cells. Dex down-regulated the receptor for interferon α/β and up-regulated JAK1 and STAT1, key components of the interferon α, β and γ pathways (Supplementary File 12a). Depletion of each of the four coregulators further enhanced the dex-regulated expression of JAK1 and STAT1 (Supplementary File 12b-e). The only coregulator-specific effect was that depletion of *CCAR2* (but none of the other three coregulators) prevented the down-regulation of the interferon receptor by dex. A heat map of the top canonical pathways represented in the dex-regulated gene set and in the coregulator-modulated gene sets can be found in Supplementary File 13.

Lastly we analyzed the dex-regulated and coregulator-modulated genes using IPA to identify candidate upstream regulators whose actions were predicted to be most highly affected (Supplementary File 14). Although the details of the coregulator effects on each upstream regulator are beyond the scope of our current discussion, we note that the pattern for the effect of *CCAR1* depletion was almost completely opposite to the pattern of effects observed for the other three coregulators, consistent with the predicted effects on the TNFR2 and acute phase response pathways (Figure 6; Supplementary File 11). This unbiased analysis further supports the conclusion that the gene-specific actions of coregulators may correlate with specific physiological pathways. Similar upstream regulator analysis was not performed on blocked genes due to the small number of affected genes.

Discussion

Gene-specific actions of coregulators

Previous published studies from a number of laboratories that examined the effects of individual coregulators on steroid hormone-regulated gene expression indicated that coregulators act in a gene-specific manner and are required for hormonal regulation of only a subset of all hormone-regulated genes [1, 5–10]. These results also suggested that different coregulators support the regulation of different sets of hormone-regulated genes, but direct comparisons of multiple coregulators have not been reported, to our knowledge. In the study reported here we conducted a direct, unbiased and genome-wide comparison of the gene-specific roles of four different coregulators on glucocorticoid-regulated gene expression.

Our results extend our knowledge of the gene-specific actions of coregulators in a number of ways: 1) In a previous study [9] with Hic-5 in U2OS osteosarcoma cells, we identified three classes of dex-regulated genes, based upon the effect of Hic-5 depletion: dex regulation modulated by Hic-5 (Hic5-modulated genes), dex regulation blocked by the coregulator (blocked genes), and Hic-5-independent genes (independent). Here, by examining four different coregulators we show that these classes (including the novel blocked class) are common to coregulators in general, indicating that multiple coregulators share this function. 2) As previously reported each coregulator has both positive and negative effects on the expression of different dex-regulated genes [9, 10, 24], but we show here that the ratio of positive to negative effects varies with the specific coregulator. 3) Likewise, each coregulator can support the actions of the hormone on some genes and oppose dex action on other genes, but the specific ratio of these

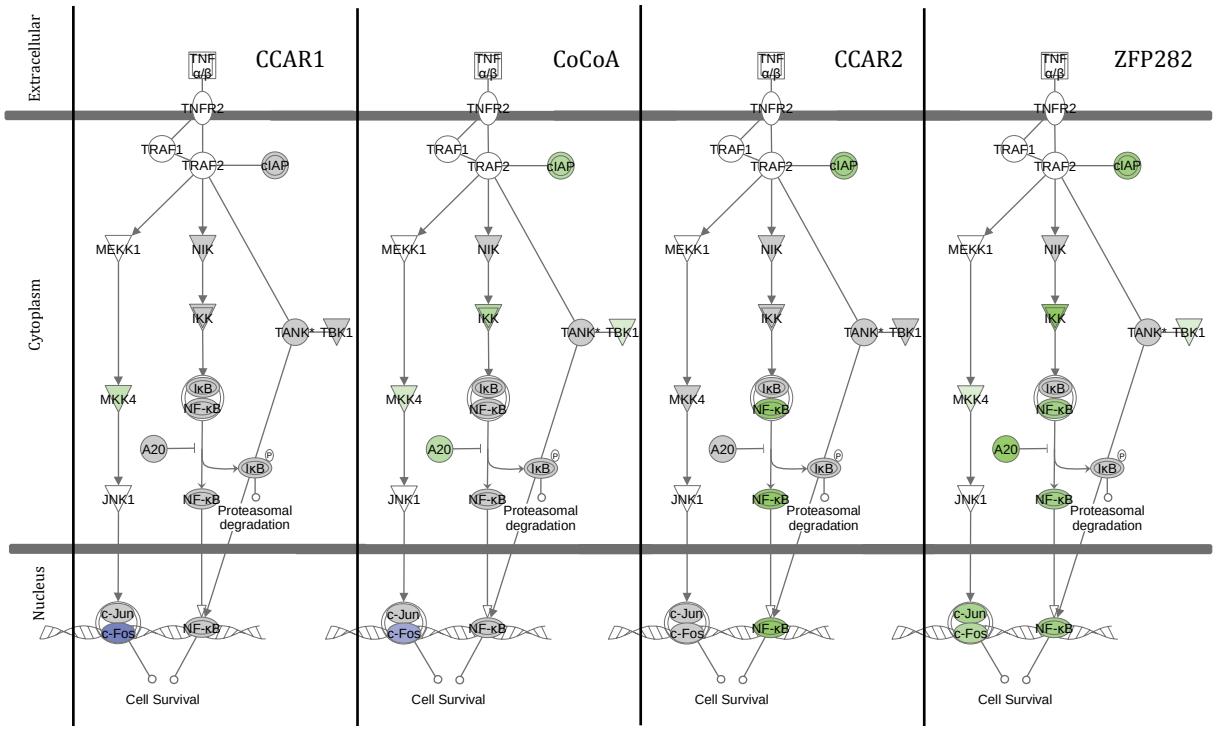

Figure 6. IPA canonical pathway analysis of the TNFR2 pathway. White genes are not regulated by dex. Grey genes are dex-regulated genes with no change in expression in dex-treated cells after coregulator depletion. Blue genes are dex-regulated genes that have lower expression in dex-treated cells after coregulator depletion, with intensity corresponding to degree of down-regulation. Green genes are dex-regulated genes that have higher expression in dex-treated cells after coregulator depletion, with intensity corresponding to degree of up-regulation. Scores representing –log(p-value) from Fisher's exact test are: 0.62 (CCAR1), 3.74 (CoCoA), 0.71 (CCAR2), 5.11 (ZNF282). The direction of dex regulation of these genes is shown in Supplementary File 10.

effects is also coregulator-specific. We thus quantify the extent to which each coregulator functioned as a coactivator (supporting gene activation by dex/GR), a corepressor (supporting gene repression by dex/GR), an anti-activator (opposing gene activation by dex/GR) or an anti-repressor (opposing gene repression by dex/GR). 4) We show that even when pairs of coregulators have extensive structural homology or have demonstrated physical and functional interactions (in transient reporter gene assays), they influence the dex-regulated expression of quite different sets of genes. For each of the four coregulators examined, one-third of the modulated genes they influence were unique to that coregulator, and another third of the modulated gene set was shared with only one of the other three coregulators. 5) In silico pathway analysis indicated that different coregulators can have a dramatically different influence on different physiological pathways regulated by glucocorticoids (Figure 6; Supplementary Files 10-14). Altogether, our findings demonstrate by direct comparison that, although there was some overlap, each coregulator influences the expression of a unique subset of dex-regulated genes. Furthermore, our results support the hypothesis that the gene-specificity of coregulators has important physiological implications.

The proportion of shared regulated genes among the four coregulators was quite low with many genes being regulated in a coregulator-specific manner (Figure 2). The structural homology between *CCAR1* and *CCAR2* does not increase the proportion of genes with shared regulation, compared to other gene pairs. Of the 216 *CCAR1*-modulated genes, the overlap with other sets of modulated genes were 59 for *CCAR2*, 55 for CoCoA, and 89 for *ZNF282*; of the 29 *CCAR1*-blocked genes, 2 were shared with *CCAR2*, 2 with CoCoA, and none with *ZNF282* (Figure 2). A small number of genes that required two structurally related coregulators has also been shown before for CBP and p300 [25,26].

The mechanistic explanation for why different genes require different coregulators for their dex-regulated expression presumably lies in the unique regulatory context of each gene. When studying hormone-regulated gene expression, regulation can occur at multiple levels. GR, as well as other DNA-binding transcription factors, can bind to a related but extremely diverse set of motifs located within enhancer and silencer elements, and each transcription factor can regulate different sets of genes in different cell types. GR can recruit different combinations of coregulator proteins to its binding

sites in the regulatory elements (enhancers and silencers), and each element also has different requirements for the types of coregulators required for dex-regulated expression of the associated gene (Figure 7). Whether or not a coregulator was recruited by GR to a regulatory element and required for dex-regulated expression of the associated gene will depend on the regulatory context of each regulatory element, because each regulatory element exists in a unique environment dictated by several different factors: GR binding site sequence, which influences GR conformation; binding of transcription factors to nearby or distant interacting sites, which can contribute to the complement of coregulators recruited to the site; and local chromatin structure, which can help to dictate the specific coregulators required to establish an appropriate chromatin environment (open or condensed) for the positive or negative regulatory actions directed by GR. Our results reflect the diversity of regulatory environments and the resulting diversity of coregulator requirements among the dex-regulated genes.

Validation of the coregulator-modulated, blocked, and independent classes of dex-regulated genes

For all four coregulators, we identified genes that fall into the coregulator-modulated, coregulator-blocked, and coregulator-independent classes of dex-regulated genes, consistent with our previous observations of these three classes for the action of the coregulator Hic-5 in dex-regulated gene expression in U2OS cells [9]. The coregulator-modulated genes for each of the four coregulators in this study represented 16-29% of all genes that were regulated by dex without coregulator depletion (Figure 1a-b, compartments iii + vii compared with comparison 1), and more than one-third of each coregulator-modulated gene set were not shared with any of the other three coregulators. This suggests that each coregulator influences a distinct portion of the biological response to dex. The blocked gene sets were considerably smaller than the modulated sets, but nevertheless we find that this class was common to all of the coregulators tested, indicating that each coregulator prevented dex-regulation of a specific set of genes. While the mechanisms through which *CCAR1*, *CCAR2*, CoCoA, and *ZNF282* block efficient dex regulation of genes remain to be determined, we showed previously that blocking of dex regulation of genes by Hic-5 involved interference with GR binding on DNA sites and chromatin remodeling at those sites [9]. Our meta-analysis of ENCODE data from A549 cells indicates that there was an increased frequency of the repressive histone mark H3K27me3 at the TSS of coregulator-blocked genes compared with all dex-regulated genes, and a decreased frequency of the active histone modification H3K4me1 (Figure 3). These findings provide clues for potential regulatory mechanisms.

While the dex-regulated gene set contained genes with very robust and modest fold-changes in response to dex, the blocked genes universally displayed modest fold changes upon hormone treatment (Supplementary File 9). A limited degree of regulation by dex could be an inherent property of the blocked gene set, or it could be attributed in part to incomplete depletion of the coregulators (Supplementary File 7). However, we note that depletion of each of the four coregulators produced a robust biological effect in terms of the number of coregulator-modulated genes.

Direct comparisons, such as the one performed here, are required to compare properly the effects of depletion of different coregulators. Nevertheless, as a generalization we note that the numbers of genes in the coregulator-modulated class were similar among the four coregulators studied here and two previously studied coregulators, Hic-5 [9] and G9a [10]. However, many more blocked genes were observed for Hic-5 than for the four coregulators studied here. Hic-5 depletion caused all-or-none effects on the hormonal regulation of many dex-regulated genes, while the effects of depleting *CCAR1*, *CCAR2*, CoCoA, *ZNF282*, and G9a generally resulted in less dramatic changes in the hormone response of individual genes.

Differential modulation of glucocorticoid-regulated physiological pathways by individual coregulators

Our pathway analyses of the modulated gene sets for four coregulators indicated that *ZNF282*, CoCoA, and *CCAR2* were all involved in supporting various aspects of the anti-inflammatory actions of dex. Thus, although among these three coregulators there is a relatively low percentage of overlap in the dex-regulated genes they control, they cooperate in facilitating dex regulation of a number of important inflammatory and anti-inflammatory genes (Figure 6 and Supplementary Files 10-14). However, depletion of *CCAR1* had no effect on dex regulation of genes in TNFR2 and acute phase pathways (Figure 6; Supplementary Files 10-11). In contrast, all four coregulators had similar actions on the interferon signaling pathways (Supplementary File 12), consistent with the notion of pathway-specific actions of GR coregulators.

Our results for *CCAR1* in this study extend our previous analysis of the pathway-specificity of this coregulator. We previously tested the effects of depleting 10 different coregulators on the ability of dex to induce expression of several adipogenic genes in the 3T3-L1 preadipocyte cell line and several anti-

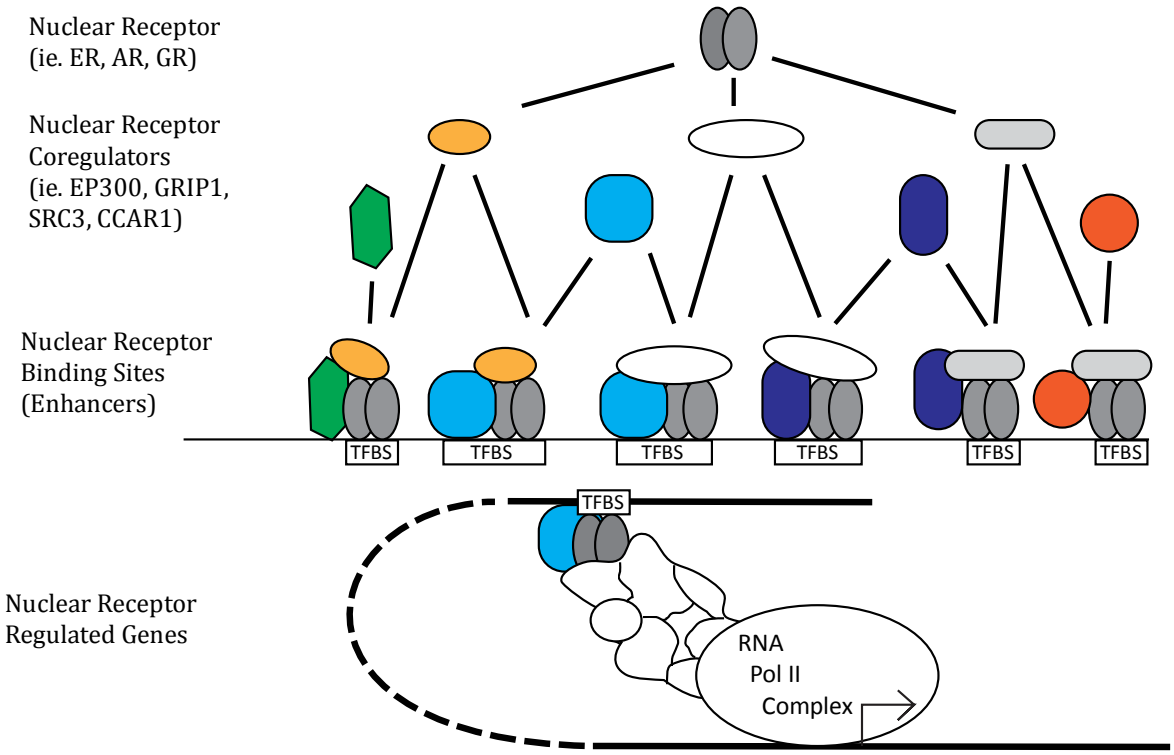

Figure 7. Regulation of gene expression occurs at multiple levels. In our model system, nuclear receptors bind to enhancers after hormone treatment and recruits coregulators. Different coregulators are recruited to (and/or required for) each enhancer and each enhancer regulates one or a few target genes. Coregulators can serve as an intermediate regulatory step for hormone regulation through differential recruitment to and requirement for enhancers as well as having both coactivating and corepressive effects.

inflammatory genes in A549 cells [18]. *CCAR1* and each of the nine other coregulators had distinct patterns of action on the adipogeneic and anti-inflammatory genes, with *CCAR1* exhibiting the strongest differential specificity among these two pathways. *CCAR1* was required for dex-induced expression of adipogenic genes but not for induction of the anti-inflammatory genes by dex. We also found that *CCAR1* was required for differentiation of the 3T3-L1 cells to mature adipocytes in response to an adipogenic cocktail that includes dex. Combined with our current findings, these two studies indicate that *CCAR1* was important for dex actions in adipogenesis but not for anti-inflammatory actions of dex. These results suggest that *CCAR1* may prove to be an attractive target to prevent some side effects of glucocorticoids when employed clinically for extended treatment regimens.

Glucocorticoids regulate thousands of genes in various cell types, with different subsets of these genes belonging to the different glucocorticoid-regulated physiological pathways. If the gene-specific actions of coregulators do indeed correlate with specific physiological pathways, as suggested by our data and several previous studies, then regulating the activity of specific coregulators provides a potential mechanism for the cell to modulate the hormone response. This could be accomplished by modulation of signaling pathways that regulate the protein levels, protein-protein interactions, or post-translational modifications of specific coregulators. For the same reason, coregulators could prove to be attractive therapeutic targets for ameliorating side effects of hormone therapy.

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Supplementary Material

Supplementary Files 1-14. See the electronic version of this article for Supplementary Material at www.nrsignaling.org/nrs12002.

Public Datasets

Gene Expression Omnibus

GSE58715

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